Endodontic drug delivery for root surface disinfection: a laboratory feasibility evaluation

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Abstract: OBJECTIVES This study aims to assess the potential of a mixture of three antibiotics (TreVitaMix, TVM) as an intracanal dressing to disinfect the outer root surface by applying a new in vitro model. MATERIALS AND METHODS Fifty freshly extracted bovine roots were endodontically treated. Forty samples were then thoroughly scaled, mounted to petri dishes, gas sterilized, and randomly allocated to four groups (n = 10/group) according to their intracanal medication: sterile saline (NaCl; control, A); the TVM carrier material alone, i.e., propylene glycol (PG; B); TVM (C); and calcium hydroxide (D). In an additional group (E), the cementum was not removed and TVM was placed. Petri dishes were filled with Fastidious Anaerobe Agar, inoculated with Fusobacterium nucleatum suspension and then anaerobically incubated during 48-h intervals at 37 °C up to 192 h. Inhibition zones around the roots were then measured after each incubation period (mm(2)). RESULTS Only teeth inoculated with the TVM dressing showed inhibition at all time points, whereas the other treatments showed no peri-radicular growing inhibition. Presence of cementum had no negative effect on disinfection (p = 0.9320). CONCLUSION TVM was able to penetrate through the dentine and inhibit the bacterial growth of F. nucleatum up to 192 h. CLINICAL RELEVANCE TVM might have the potential to sustainably disinfect the outer root surface in perio-endo lesions and serve as an adjunctive antimicrobial agent.

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Endodontic drug delivery for root surface disinfection – a laboratory feasibility evaluation

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Key words: agar diffusion, bacteria, dentine, in vitro, TreVitaMix, perio-endo lesion

Declaration of interests:

The authors declare they have no conflicts of interest.

Abstract

Objectives To assess the potential of a mixture of three antibiotics (TreVitaMix, TVM) as an intra-canal dressing to disinfect the outer root surface applying a new *in vitro* model.

Materials and Methods Fifty freshly extracted bovine roots were endodontically treated. Forty samples were then thoroughly scaled, mounted to petri dishes, gas sterilized and randomly allocated to four groups (n=10/group) according their intracanal medication: Sterile saline NaCl (control, A), the TVM carrier material alone, i.e. propylene glycol (PG; B), TVM (C) and calcium hydroxide (D). In an additional group (E), the cementum was not removed and TVM was placed. Petri dishes were filled with Fastidiuos Anaerobe Agar, inoculated with *F. nucleatum* suspension and then anaerobically incubated during 48 h intervals at 37°C up to 192 h. Inhibition zones around the roots were then measured after each incubation period (mm^2).

Results Only teeth inoculated with the TVM dressing showed inhibition at all time points, whereas the other treatments showed no peri-radicular growing inhibition. Presence of cementum had no negative effect on the disinfection ($p = 0.9320$).

Conclusion TVM was able to penetrate through dentine and inhibit the bacterial growth of *F. nucleatum* up to 192 h.

Clinical relevance TVM might have the potential to sustainably disinfect the outer root surface in perio-endo lesions and serve as an adjunctive antimicrobial agent.

Introduction

Several etiological factors contribute to the development and progression of endodontic and periodontal diseases. However, the primary cause of both diseases is the presence of bacterial infection [1-5]. Studies suggest that infected root canal systems and periodontal pockets have similar microbiological flora [2, 4-8] and it is widely accepted that untreated infections of one of these tissues can lead to signs or symptoms of disease initiation and progression within the other compartment [6, 7, 9, 10]. This can occur in either direction, for example from the root canal to the periodontium and *vice versa*. Potential pathways for communication are dentinal tubules, accessory canals, canal ramifications, apical deltas, fins, and transverse anastomoses. Since bacteria have been shown to progress from one compartment to the other this might be also true for medicaments applied to the compartments.

In general, there are more microbes and more species in periodontal pockets than in infected root canal systems, and the microflora in infected root canals of teeth that have concurrent endodontic and periodontal diseases is more complex than in teeth with pathosis confined to the periapical region only [4]. One reason for this is that medicaments applied to periodontal pockets show a very short sustainability due to anatomical factors and clearance [11]. In contrast intracanal medicaments show a higher substantivity [12]. The treatment of periodontal infections is challenging, therefore the root canal might be considered as a targeted space for the placement of a medicament to disinfect the pathologically altered tissues, in both compartments. The antimicrobial agents used as inter-appointment dressings in combined lesions should theoretically be able to penetrate through the dental tissues. Desirably, this would lead to a sufficiently high antimicrobial concentration able to eliminate the disease-causing bacteria in a predictable manner in both compartments [13, 14]. To date, the most predictable antiseptic agent remains calcium hydroxide [15], which has been reported to be able to penetrate through the peripheral dentine [16]. Due to the complexity of root canal infections, however, it is unlikely that any single medicament results in an effective and predictable disinfection of the root canal complex, especially if antibiotic dressings are used [17, 18].

Hoshino and co-workers determined that a combination of ciprofloxacin, metronidazole and minocycline was able to disinfect infected root dentine *in vitro* [17]. Sato et al. found that this

combination was sufficient to disinfect infected root dentine *in situ* [18]. Especially metronidazole showed the ability to penetrate through the dentine [19].

A modified antibiotic mixture consisting of ciprofloxacin, cerfuroxim and metronidazol (TVM; TreVitaMix, MedCem GmbH, Weinfelden, Switzerland) was further developed and later on primarily used for revascularisation in pediatric dentistry [20-26]. Such an antimicrobial agent could be an alternative way to sustainably reach pathogenic bacteria of the periodontium, provided that an adequate permeation through the dentine system is achieved. Unfortunately, the specific application of intracanal dressings as an alternative administration route to treat periodontitis has not been well investigated. Therefore, this study aimed to assess this commercially available mixture of three antibiotics (TVM) and their ability to penetrate through dentine to inhibit bacterial growth, exemplarily evaluated using *Fusobacterium nucleatum,* in a newly developed agar diffusion set-up.

The hypothesis was that TVM will inhibit bacterial growth by means of diffusion through the root structure of bovine teeth and that the cementum will not impair the postulated antibacterial effect.

Material and methods

Sample preparation

In this *in vitro* study, the agar diffusion test was specifically modified to allow study of the antimicrobial effect of TVM through bovine roots and to compare this effect to calcium hydroxide.

For this purpose, fifty freshly extracted bovine teeth were selected and the roots carefully inspected for cracks and lateral canals using a stereo-microscope (Stemi 2000, Carl Zeiss, Oberkochen, Germany). The roots were randomly allocated into five experimental groups (n=10 each): In four groups (A-D), the roots were thoroughly scaled with curettes (Hu-Friedy, Chicago, USA) to remove the cementum and subsequently rinsed with tap water, whereas in one group (E), the cementum remained intact. In all groups the crowns were removed 2 mm below the cemento-enamel junction using a water-cooled diamond saw (Diamand Wafering Blade 100 x 0.3 x 12.7 mm, Buehler GmbH, Düsseldorf, Germany), and the coronal third of the root was widened with Gates Glidden Drills size 4 and 3 (Dentsply, Ballaigues, Switzerland). The working length was measured with an ISO 10 K-File (Dentsply, Konstanz, Germany) by inserting the file into the canal until the tip was just visible beyond the apex.

The working length was determined by subtracting 1 mm from the measured length. Afterwards, the root canals were instrumented using ProTaper® Universal up to F3 and ProFile® (Dentsply) size 45, .04 taper at full working length. To prevent contamination of the outer root surface with endodontic irrigants during instrumentation, the apical part of the root was sealed with a flowable composite (Tetric Evo Flow, Ivoclar Vivadent, Schaan, Liechtenstein) and a rubber dam was applied to the coronal aspect of the root (Fig. 1a). The root canals were irrigated with 1 ml of a 1% NaOCl solution (Hedinger, Stuttgart, Germany) after each instrument [27], using a 30-gauge irrigating needle (Hawe Neos, Bioggio, Switzerland) 1 mm short of working length. In total the root canals were irrigated cumulatively for 25 min with NaOCl followed by a final flush of 5 ml of 17% EDTA (Kantonsapotheke Zürich, Zürich, Switzerland) for 5 minutes to remove the smear layer. Finally, the canals were rinsed with 10 ml 0.9% sterile saline (NaCl, Braun Melsungen, Melsungen, Germany) to remove the EDTA. Thereafter, specimens were cut coronally to a final root length of 10 mm and the composite at the apical part was removed with a scaler.

Agar diffusion test

The roots were inserted in a prefabricated notch of a sterile petri dish (Sterilin Limited, Newport, UK) and fixed with sticky wax (BELLADI RUSCHER GmbH, Altnau, Switzerland) (Fig. 1b). The apical foramen as well as the exposed root was completely covered with sticky wax to avoid dehydration and subsequent crack formation. Thereafter, sterile saline was applied into the canals and petri dishes for rewetting and keeping the specimens moist. All samples were then welded into sterile foil (steriCLIN, Vereinigte Papierwarenfabrik GmbH, Feuchtwangen, Germany) and gas sterilized with ethylene oxide at 37°C for 5 h duration (Steri-Vac^{TM}4XL, 3M, St. Paul, MN, USA).

Fusobacterium nucleatum subsp. nucleatum (OMZ-Nr. 598) was grown anaerobically on Columbia Blood Agar plates (CBA) (Difco BD ® 279240, DIFCO-BD - Sparks, MD, USA; Oxoid, Ltd., Basingstoke, Hamps, UK) supplemented with 5% (v/v) hemolyzed human blood for 2 to 3 days at 37°C, then transferred to a modified fluid universal medium mFUM [28] containing 76 mmol/l Sörensen's buffer (final pH 7.2) and 0.3% of glucose, and anaerobically incubated at 37°C with 5% CO_2, 10% H_2, and 85% N_2 (Microincubator MI22NK, Scholzen Microbiology Systems AG, Necker, Switzerland).

FAA-Agar (acumedia®, NEOGEN® Corporation), which was autoclaved (121°C, 20 min, 1.2 bar) was transferred into a preheated water bath (50°C) (Typ: Haake C 10, Thermo Electron, Karlsruhe, Germany) until its use. The density and turbidity of the liquid *F. nucleatum* culture was controlled under a microscope (Leitz Dialux 22, Leica Microsystems AG, Glattbrugg, Switzerland) and the optical density (OD) adjusted at the wavelength of 550 nm to $OD_{550nm=}$ 1.00 ± 0.05 (Spectrophotometer U-2000, HITACHI, Tokyo, Japan), which corresponds with 10^8 colony-forming units (CFU) per mL.

The gas sterilized specimens were then unwrapped and dried with sterile paper points under a laminar flow clean bench (SKAN EVZ 120, SKAN AG, Allschwil, Switzerland) before insertion of the intracanal dressings with a Lentulo (Dentsply) as follows:

In group A, sterile saline only was applied as a control. The pure carrier material, propylene glycol (PG), was placed in group B and in groups C and E the triple antibiotic paste (TVM), which contained ciprofloxacin (20 mg), cerfuroxim (40 mg) and metronidazol (40 mg) was mixed with added macrogolum unguentum (PG) to viscous consistency with the ratio 1:2 respectively 100 mg of TVM were mixed with 200 mg of PG and applied into the canals. In group D, a mixture of calcium hydroxide ($Ca(OH)_2$) (Merck KgaA, Darmstadt, Germany) and sterile NaCl solution was placed.

After placement of the intracanal dressings, the root accesses were sealed with a dual curing self-adhesive cement (Clearfil SA Cement, KURARAY MEDICAL INC., Tokyo, Japan) and immediately light cured for 20 s (mode: HIP, 1200 mW/cm2, Bluephase, Ivoclar Vivadent).

Thereafter, 1 ml of *F. nucleatum* suspension was evenly spread into every petri dish and 30 ml FAA-Agar were added using an electronic pipettor (Esypet, Eppendorf, Hamburg, Germany) and serological pipettes size 1 ml and 25 ml (Serological pipette, SARSTEDT, Nümbrecht, Germany). The petri dishes were carefully shaken to facilitate dispersion of the bacterial suspension and liquid FAA-Agar. After the agar solidified, all petri dishes (groups A - E) were covered with lids and anaerobically incubated (Microincubator MI22NK) at 37°C for 48 h. The applied petri dish model design is illustrated in Figure 2. The initail FAA- Agar was removed after the 48 h incubation period and groups A - E were then again inoculated with new *F. nucleatum*-Agar as described above and incubated for

another 48 h up to 96 h. In groups A to D this procedure was repeated two more times up to 144 h, respectively 192 h.

Analysis

After each incubation cycle, standardized photographs were taken of the specimens to measure their respective inhibition zones (Fig. 1 c) (Nikon D90, Nikon Coorporation, Tokyo, Japan) using a scaled camera stand (RS1 Camera Stand, Kaiser Fototechnik GmbH & Co.Kg, Buchen, Germany).

All images were analyzed with a computer software (ImageJ 1.42q, Wayne Rasband, National Institutes of Health, USA) and the area in mm^2 of the outer and inner contour was determined and the difference calculated.

Differences between time measurements and groups were tested by one-way analysis of variance (ANOVA) and Scheffé post-hoc test ($p < 0.05$). Additionally, the comparison between scaled (group C) and non-scaled group (group E) at 96 h was analyzed using Mann-Whitney U-test.

To exclude potential material leakage through the roots despite the embedding process, specimens were checked with a dye penetration test after completion of the experiments. For this purpose, roots were covered with nail varnish leaving the apical and ? exposed (Malva, Geneva, Switzerland), embedded in epoxy resin (Paladur, Heraues Kulzer, Hanau, Germany) and subsequently placed for 20 h into a 0.5% fuchsin-solution (Fluka, Buchs, Switzerland). Afterwards, the roots were cut longitudinally with a diamond saw (Isomet 1000, Buehler Ltd., Lake Bluff, IL, USA) under kerosene cooling (Fluka) and fuchsin penetration was assessed using a light-microscope (Tessovar, Carl Zeiss, Feldbach, Switzerland).

Results

The inhibition zones (in mm^2) of groups A-D after 48 h, 96 h, 144 h and 192 h are presented in Table 1. Only the test groups with the antibiotic mixture showed inhibitions zones at all time points, which were 723.1 ± 412.0 mm^2, 711.3 ± 412.3 mm^2, 673.3 ± 192.5 mm^2 and 652.3 ± 150.0 mm^2, respectively. All other groups in the scaled samples showed no growth inhibition effects at any time point.

The comparison of the samples after an incubation time of 96 h with removed (group C) and intact cementum (group E) showed comparable inhibition zones of 711.3 ± 412.3 mm^2 and 724.0 ± 219.0 mm^2 ($p = 0.9320$), respectively.

The fuchsin penetration test showed no leakage at the occluding composite interface at all specimens. Thus, any leakage of the compound is presumed to occur through the root dentin walls.

Discussion

This study aimed to evaluate the ability of a mixture of three antibiotics (TVM) to penetrate through root dentine and to inhibit the growth of *Fusobacterium nucleatum* using a modified agar diffusion model up to 192 h *in vitro*.

In this study, bovine teeth were used as they have been the most widely used substitute for human teeth in dental studies and are easy to obtain in large quantities, good condition and are of a more uniform composition than that of human teeth [29]. Although bovine teeth are commonly used, some concerns about the application of data obtained from bovine to human teeth have been raised, as their chemistry and structure are not fully identical [30, 31] and inconsistent data exists for microleakage of bovine dentine compared to that of human dentine [32, 33]. These different anatomical and morphological aspects should be considered. Despite these limitations, bovine teeth were used in this comparative feasibility study because other limiting factors when using human teeth could be avoided. In this context, Vasiliadis and co-workers [34] showed in human teeth that intratubular calcification was more pronounced at the mesial and distal root aspects than in the bucco-lingual counterparts. Paqué et al. [35] reported that dentine sclerosis was the main factor influencing penetrability of root dentine. It is a physiological phenomenon that starts in the third decade of life in the apical root region and advances coronally with age. The degree of sclerosis might therefore have a great impact in dentine permeability. Further research on this topic is necessary because the incidence of periodontitis increases with age. Additionally scaling and root planing as well as supportive periodontal therapies could also influence, over time, the amount of sclerotic dentine.

In this study, the carrier material propylene glycol PG was used in combination with TVM to achieve a faster antimicrobial penetration through the dentine [36]. All specimens treated with TVM showed

clear inhibition zones up to 192 h and therefore proved the ability of TVM to penetrate through dentine.

No differences in the inhibition zones were observed between scaled (group C) and non-scaled (group E) specimens. This is in accordance with the results reported by Gomes et al. [37], where no differences were found between presence or absence of cementum when using 2% CHX as an intracanal antimicrobial.

Further, the smear layer caused by scaling was left intact on the root surfaces. In this in-vitro study, because our focus was not the removal of the smear layer, only a tap water rinse was used after scaling. A complete and efficacious removal of the smear layer with the treatment used is not given [38]. For root conditioning and smear layer removal EDTA [39], citric acid [40] and tetracycline hydrochloride [41] is recommended but these root conditioning procedures are normally used in regenerative periodontal therapy or recession treatment [42]. In clinical periodontal treatment root conditioning after scaling and root planing is not common. In this in-vitro study, we simulated clinical conditions and rinsed the root only with tap water to remove the debris after scaling. Due to this fact, we also isolated the outer root surface to avoid EDTA contact with the root surface during endodontic irrigation. It must be assumed that the scaled roots have more open dentine tubules [43, 44] and therefore more of the antibacterial medicament could diffuse from the inner to the outer surface and result in a greater standard deviation, as presented in the results. Possibly, the measurable antimicrobial diffusion would be increased by root conditioning and show statistical significance.

In the present study, calcium hydroxide was used as a control medicament, because it represents the mostly investigated intracanal disinfectant [15]. It has been shown that this dressing needs to be in the root canal for at least 3-4 weeks in order to diffuse throughout the dentine and achieve effective pH values [16]. In this study, however, calcium hydroxide was left into the canal for 192 h only. Consequently, the full antimicrobial effect of calcium hydroxide was probably not yet reached and therefore no bacterial inhibition could be observed. Nerwich et al. [16] also reported in their study that it took nearly seven days for the pH to rise to 9.0, a level at which many bacteria do not grow. However, after 8 days no effect was detectable, which corroborates the findings of the present study.

One advantage of the model used for this investigation was that the apex was positioned outside of the petri dish and covered with sticky wax. Therefore, potential medicament leakage from the apical delta could be excluded. In addition, the volume of the agar was limited to 30 ml, so that the level of the agar was always about 1–2 mm below the end of coronal end of the cut root, thus preventing any leakage as well. However, in order to prove that the penetration of the medicament was indeed limited to the transdentinal pathway and not through the fillings at both ends of the test set-up, a dye leakage test was performed. None of the samples showed fuchsin leakage.

In this first feasibility study, *F. nucleatum* was selected as target microorganism due to the fact that it is a major co-aggregation bridge organism linking early and late colonizers [45] and is common in the subepithelial periodontal biofilm as well. The evaluation of other bacteria and more complex biofilms is of course also mandatory in future studies.

Never the less, based on our preliminary findings,one may postulate that the use of a triple antibiotic medicament might help in eliminating bacteria in the periodontally affected compartment, especially because a wide variability in periodontal pathogen antibiotic resistance pattern can be found, which is of critical concern to clinicians when empirically selecting antibiotic treatment regimens for periodontitis patients [46-48]. In root filled and endodontic infected teeth, biofilm formation can be observed and subsequently results in a reservoir for antibiotic resistance [49]. Bacteria organized in a biofilm are 100 to 1,000 times more tolerant to antimicrobials as equivalent planktonic cells [50]. Therefore mechanical disruption of the biofilm is mandatory for successful antibacterial therapy [51]. In this study, the roots were mechanically instrumented but it is recognized that complete biofilm removal is difficult [52, 53]. A combination of antibiotics could therefore help to decrease the likelihood of the development of resistant bacterial strains [54]. It must be considered that antibiotic diffusion through dentine could lead to subinhibitory concentrations on the infected sites that might, in turn, induce bacterial resistance especially when the biofilm is intact. An adequate concentration [55] after diffusion through dentine must be guaranteed. Therefore further diffusion studies including biofilm formation are needed. A further recognized problem with the use of antibiotics, in particular tetracyclines, is tooth discoloration [56]. The triple antibiotic medicament TVM used in this study

exchanged minocycline with cefuroxime, which belongs to the class of the cephalosporine antibiotics and has been shown to no cause tooth discoloration [22].

Conclusion

Within the limits of this *in vitro* feasibility study, the triple antibiotic mixture TreVitaMix as an intracanal medicament showed the potential to quickly penetrate through bovine dentine, and to inhibit bacterial growth for up to 192 h. Therefore, the medicament might have the potential to disinfect the outer as well as the inner root surface and could be a treatment option for periodontally-endodontally involved teeth. However, more research on this topic is needed to confirm this observation.

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hours of Incubation	Groups A NaCl	B PG	C TVM	D $Ca(OH)_2$
48 h	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	723.1 ± 412.0 [a]	0.0 ± 0.0 [b]
	0.0	0.0	272.4	0.0
	0.0	0.0	1557.0	0.0
96 h	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	711.3 ± 412.3 [a]	0.0 ± 0.0 [b]
	0.0	0.0	270.7	0.0
	0.0	0.0	1569.00	0.0
144 h	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	673.3 ± 192.5 [a]	0.0 ± 0.0 [b]
	0.0	0.0	364.6	0.0
	0.0	0.0	935.9	0.0
192 h	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	652.3 ± 150.0 [a]	0.0 ± 0.0 [b]
	0.0	0.0	384.4	0.0
	0.0	0.0	838.00	0.0

Identical superscript lowercases represent – where appropriate - values, which do not differ statistically significantly from each other (ANOVA, Scheffé, read vertically).

Table 1

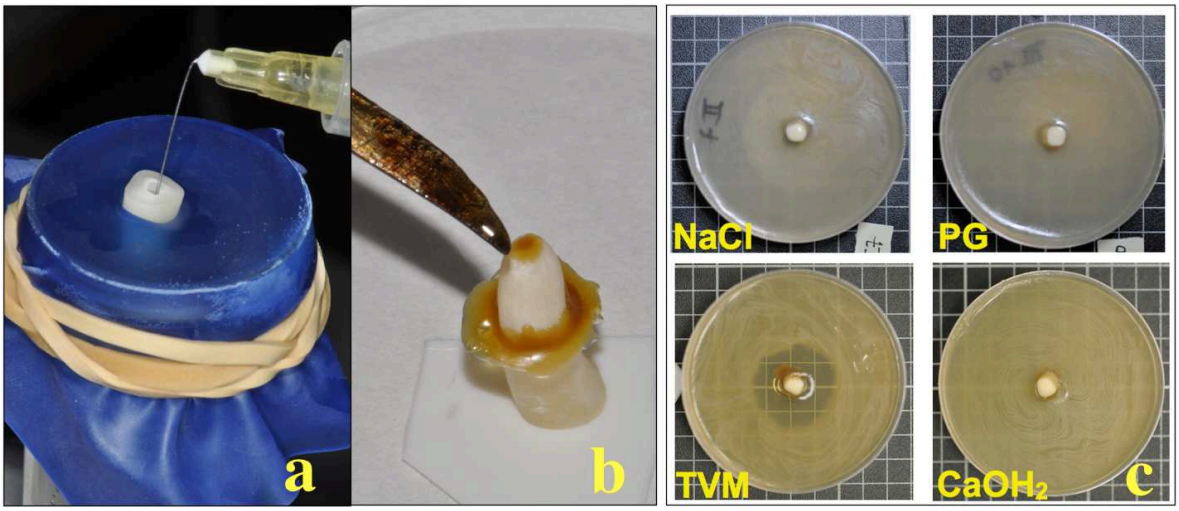

Fig. 1

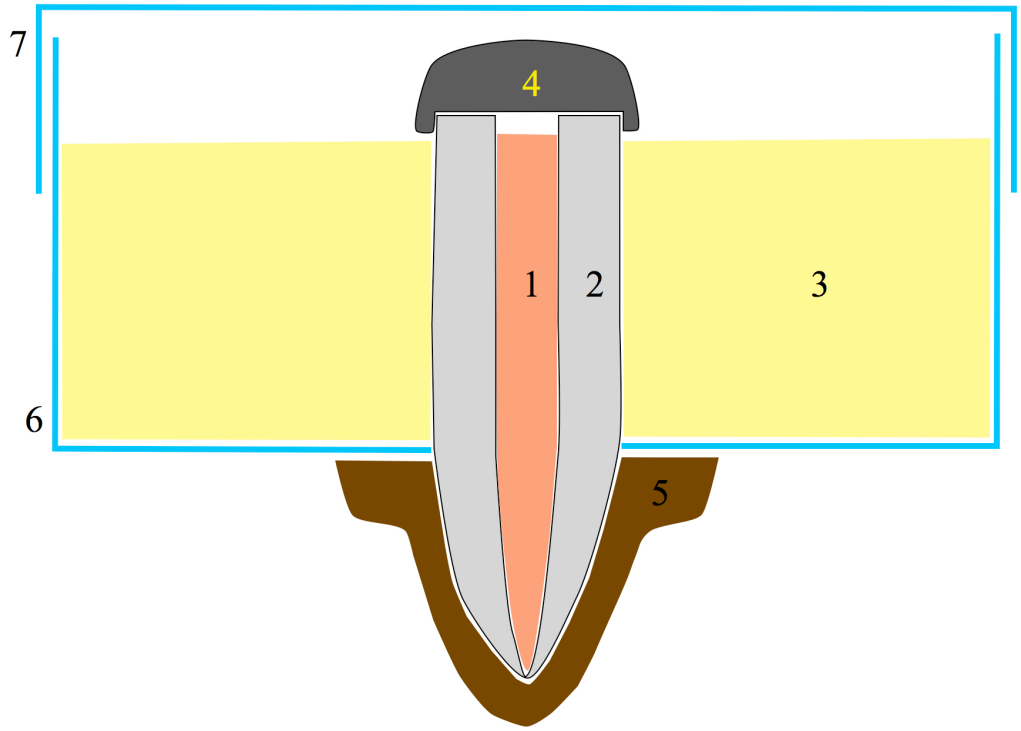

Fig. 2

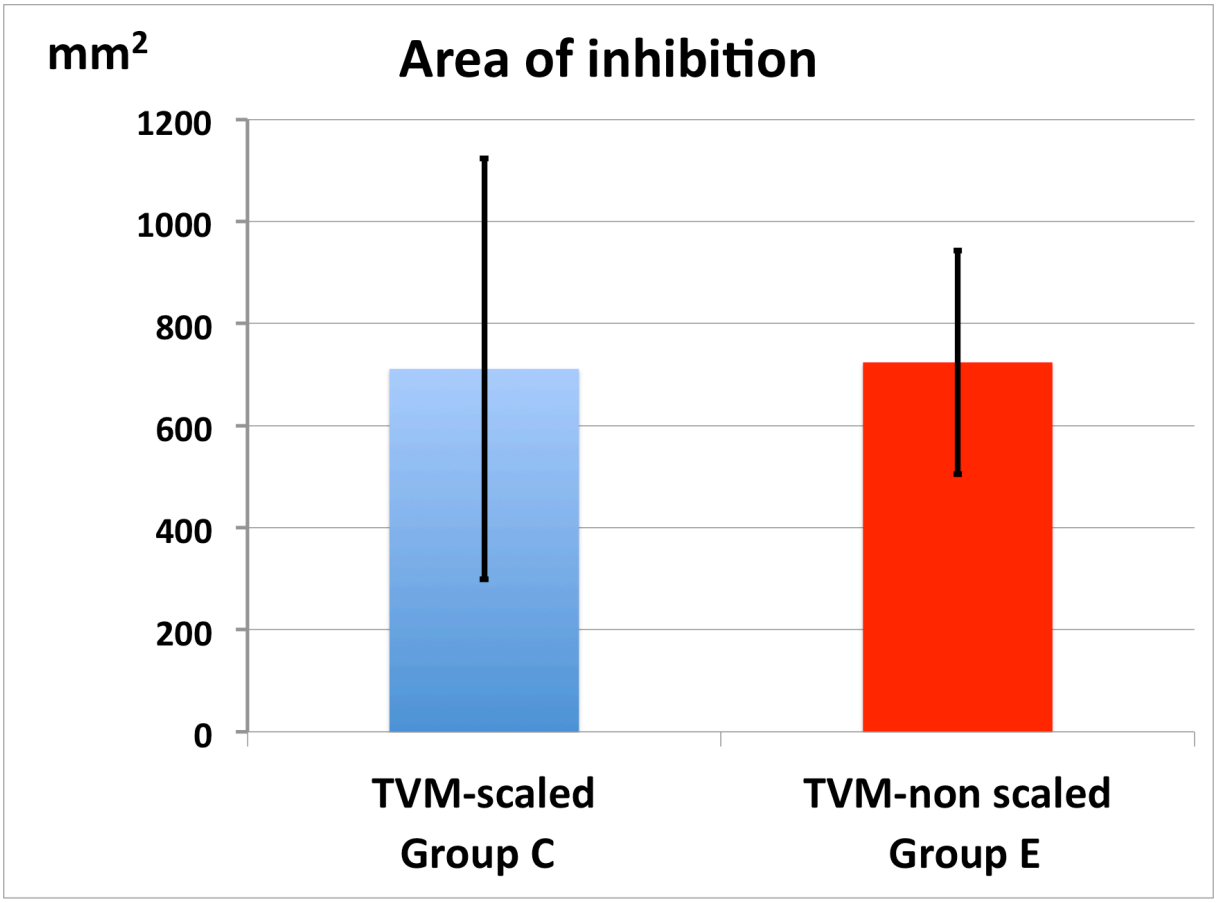
mm²
Area of inhibition
1200
1000
800
600
400
200
0
TVM-scaled
Group C
TVM-non scaled
Group E

Fig. 3

CAPTIONS

Table 1:

Comparison of mean areas of inhibition ± standard deviation and minimum respectively maximum in mm^2 for groups A-D. (NaCl: sterile saline; PG: propylene glycol,
TVM: TreVitaMix; $Ca(OH)_2$: calcium hydroxide)

Fig. 1

Root sample preparation: Disinfection of the roots using rubber dam (a), gluing of the apical part of the roots with sticky wax to a petri dish (b) and representative standardized photographs of the inhibition zones (c) of the different intracanal dressing groups (NaCl: sterile saline; PG: propylene glycol, TVM: TreVitaMix; $Ca(OH)_2$: calcium hydroxide).

Fig. 3

Petri dish model. The numbers present the different objects in the design.
1. Intracanal medicament 2. Bovine root 3. F. nucleatum inoculated Agar 4. Composite 5. Sticky wax 6. Petri dish 7. Patri dish lid

Fig. 3

Comparison of mean areas of inhibition ± standard deviation (mm^2) for groups for the TreVitaMix (TVM) treatment scaled (C) *versus* non-scaled (E).

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